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Studies of Salvinorin-Based Antagonists to Elucidate Pertinent Interactions for Kappa Opioid Receptor Antagonism

Madeline Keane
University of Mississippi

STUDIES OF SALVINORIN-BASED ANTAGONISTS TO ELUCIDATE PERTINENT INTERACTIONS FOR KAPPA OPIOID RECEPTOR ANTAGONISM

Madeline Marie Keane

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A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Approved By

Advisor: Dr. Hoang V. Le _____

Reader: Dr. Jason Paris _____

Reader: Dr. Joshua Sharp _____

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ABSTRACT

Opioid abuse, leading to addiction and related deaths, has created a chronic epidemic in the United States for the past 30 years. This crisis has sprung from reliance on the prescription of opioid analgesics as the primary method for the management of pain in the 1990's. At that time, these drugs, specifically Purdue Pharma's OxyContin, were marketed as non-addictive. Due to this systemic minimization of the addictive properties of opioid analgesics, as prescription rates increased, opioid-related mortality rates climbed. This epidemic continues to be pervasive, as opioid-related overdose resulted in 47,600 deaths in 2017. In addition to the opioid epidemic, there is mounting evidence of a psychostimulant addiction crisis, with psychostimulant-related overdose deaths increasing 37% from 2016 to 2017. It has been shown that there is potential for the treatment of psychostimulant and opioid addiction by antagonizing the kappa opioid receptor (KOR). The KOR is one of three opioid receptors involved in antinociception and thus plays a role in opioid addiction. Opioid analgesics acting on these G protein-coupled receptors lead to an agonistic effect. Selectivity for the KOR is of interest because it also plays a role in behavioral processes that contribute to addiction cycles, such as anxiety and depression. Antagonizing the KOR decreases symptoms of these stress states and leads to the reduction of drug reinstatement. Salvinorin A, a naturally-occurring hallucinogenic compound, is known to be a selective KOR agonist. Modifications of the structure of salvinorin A have yielded compounds found to exert antagonistic effects selectively upon the KOR. These modifications changed the topology of of the ring structure in these compounds; thus we suspect that these new ring structures help the compounds bind better to the inactive or active state of the KOR. This study identifies critical interactions between salvinorin-based antagonists within the KOR, achieved by coupling structure-based drug design and computational modeling. This approach will facilitate the creation of more selective compounds for antagonizing the KOR. Docking studies performed concluded that the topology of the salvinorin scaffold determines agonistic or antagonistic functionality on the KOR.

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Chapter 1

Introduction

1.1 The Opioid Crisis

The opioid crisis, officially a public health emergency under federal law, is a result of various factors and thus requires a multifaceted solution. Much of the complexity of this crisis has stemmed from the decades-long struggle to balance and standardize pain management with prevention and mitigation of addiction.

A lack of effective chronic pain relief methods and analgesics persisted into the latter half of the 20th century.[1] Consequently, opioids, commonly morphine, were the only options available. Heroin, a street drug, is derived from morphine and acts on the same receptors.[1],[2] The connection between heroin and morphine led to a rapid decrease in the number of opioid prescriptions. There was a movement of 'opiophobia' throughout the country, a panicked response that the prescription of opioids would lead to addiction.[1],[3] The resulting reduction of analgesic prescriptions, along with a lack of systemic regulation, led to mismanagement of pain, whether chronic, cancer-related, or post-operative in nature.[1],[4] American physicians asserted that there was a state of under-treatment of pain throughout the US, calling the drastic reduction of opioid prescriptions into question.[4] Typically, prescription of opioids had been exclusively reserved for malignant cancer-related pain. To combat the general lack of quality care for pain, it was suggested that the prescription of opioids be expanded to include chronic pain states.[1],[5]

In the 1990s, prescription opioid pain relievers were marketed as non-addictive. Two

influential yet scientifically inconclusive papers contributed to this claim.[3][6] One of these citations is merely a letter, which anecdotally states that opioids for pain management are not associated with addiction.[3] Therefore, there was a significant dearth of safety tests and demonstrable outcomes. More research was necessary in order to fully consider the complexity of human pain states, including physiological, psychological, and social conditions, which affect the human brain and its perception of pain.[7],[1]

Despite the reality of minimal thorough scientific research on the effects of opioid analgesics for the treatment of chronic pain, including reliable data in terms of its addictive properties, opioids became the primary method for treating chronic and cancer-related pain in the U.S. in the 1990s. Coupled with the efforts of physicians and programs like the American Pain Society's campaign for "pain as the fifth vital sign", chronic pain cases were rightly given more attention by doctors and the healthcare industry.[8]

New standards for pain management were created, with the primary modality of chronic pain treatment being the prescription of analgesic opioids, specifically OxyContin, which had been marketed as non-addictive.[1],[9] Beneficially, the regulations allowed for much-needed quantitative assessments for pain and thus essential data on the subject, but it also pushed for higher numbers of opioid prescriptions.[1],[10] This significant increase in the number of opioid analgesics prescribed nationwide was a result of dual causes. First, the Joint Commission (TJC) enstated new standards for pain relief, requiring that physicians provide quantitative data on patient satisfaction levels in terms of pain relief. Because such data is difficult to obtain given the subjective nature of pain, physicians relied on prescribing opioid analgesics to boost patient satisfaction, guaranteeing continued federal healthcare aid.[1] Second, Purdue Pharma pressured physicians to prescribe their opioid analgesic, often going to extremes of dubious legality. Their drug, OxyContin was marketed as a non-addictive, humane method to relieve pain in a safe way. Physicians feared legal consequences and being viewed as inhumane if they did not prescribe these drugs.[1],[11] Ultimately, Purdue Pharma was charged for misrepresentation of OxyContin, which by 2004 had become the leading drug of abuse after millions of dollars spent on convincing healthcare workers of its efficacy and safety.[12] Such manipulations

of doctors for the sale of unsafe drugs was unprecedented, and the company announced it would pay $10 billion to settle the 2,000 lawsuits filed against it related to the opioid crisis in 2019.[13] Due to these factors, the use of opioid analgesics escalated throughout the 2000s before Purdue Pharma's practices came to light.[12]

Year	Total Number of Prescriptions	Prescribing Rate per 100 Persons
2006	215,917,663	72.4
2007	228,543,773	75.9
2008	237,860,213	78.2
2009	243,738,090	79.5
2010	251,088,904	81.2
2011	252,167,963	80.9
2012	255,207,954	81.3
2013	247,090,443	78.1
2014	240,993,021	75.6
2015	226,819,924	70.6
2016	214,881,622	66.5
2017	191,909,384	59
2018	168,158,611	51.4

Figure 1.1: Opioid prescriptions in the U.S. from 2006 to 2018.

Adapted from the Center for Disease Control and Prevention.[14]

Though this new attitude toward prescription of opioids for pain relief afforded more effective pain control for the patient, the adverse effects of the surplus of opioid use quickly became apparent. Examples of these adverse effects include an increased number of incidents of oversedation, addiction, hyperalgesia, disabilities, endocrine and psychological co-morbidities, and mortality as a result of opioid use.[1] According to the Centers for Disease Control and Prevention (CDC), there was a trend of quadrupling incidence of drug poisoning deaths due to opioid analgesics from 1999 to 2012.[15]

Even given the escalating rate of addiction due to prescription opioids, physicians, especially surgeons, continue to prescribe them in high volume, with 240 million prescriptions in 2015.[16] This equates to nearly one prescription per adult in the U.S., but often the medication is simply unnecessary. While some doctors practice judicious prescribing procedures, many do not. Therefore, over-prescription of opioid analgesics is a key area for improvement needed to adequately address the opioid crisis in this country. Currently,

the most popular opioid analgesic prescribed is oxycodone.[16]

New forms of opioids have caused surges in opioid addiction and related deaths in the U.S. The first spike in the 1990s, was due to the previously discussed over-prescribing practices of addictive opioid analgesics. In 2010, a new spike in opioid-related deaths was due to the rise of heroin, followed by another spike in 2013, corresponding with the increased use of synthetic opioids like fentanyl and tramadol.[17],[18],[19],[20] Heroin is an illegal opioid made from morphine. Synthetic opioids mimic the effects of naturally occurring opioids like heroin and codeine but do so in much lower doses. For example, fentanyl is 50 times more potent than heroin and 100 times more potent than morphine.[20] Thus, it is easier to overdose on synthetic opioids, and more than 28,000 deaths in the U.S. were a result of synthetic opioid use in 2017.[20] Opioids in any of these forms were the cause of 47,600 deaths in 2017, six times higher than in 1999.[17] These distinctions in types of opioid use, which caused each surge in overdose rates, are essential in identifying potential methods for prevention and effective response to this evolving epidemic. Importantly, in 2013, 80% of heroin users confessed that they used prescription opioids before beginning to use heroin.[21]

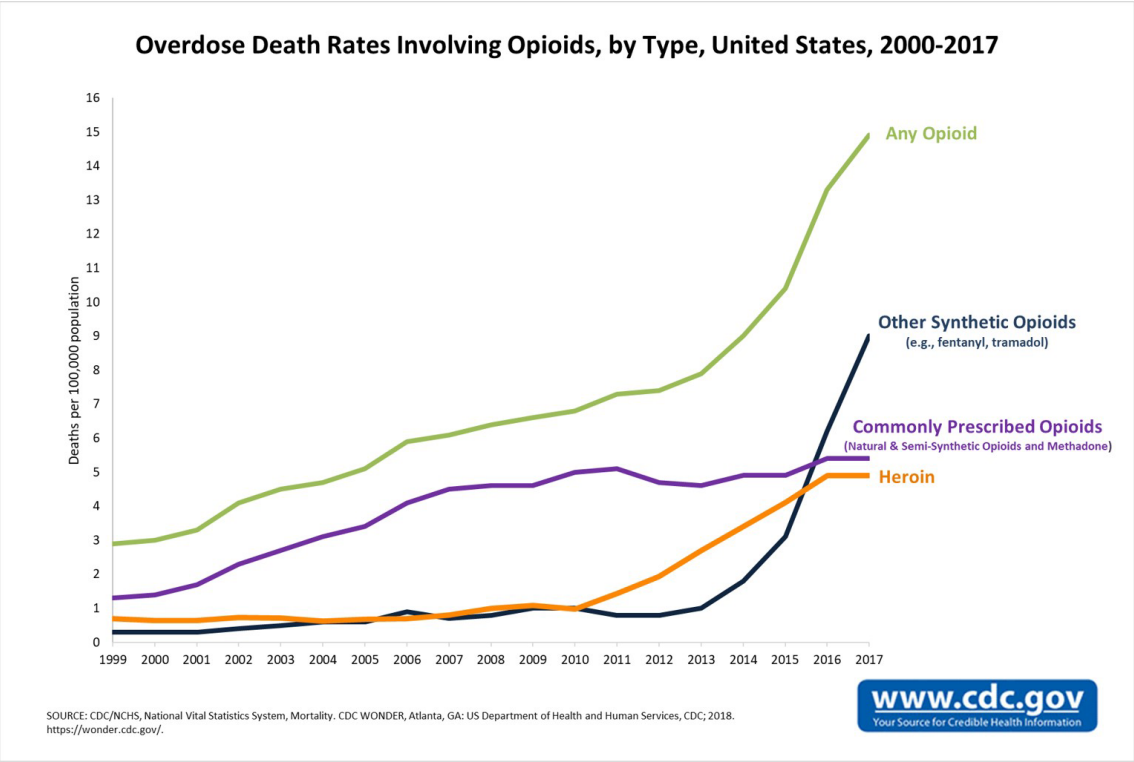


Figure 1.2: Mortality rates of overdoses involving opioids, 2000 - 2017.

Adapted from the Center for Disease Control and Prevention.[20]

The condition in which the misuse of opioids leads to clinically significant impairment and distress is defined as opioid use disorder (OUD) in the Diagnostic and Statistical Manual of Mental Disorders (DSM) V.[22] Current treatments for OUD have significant issues, such as high discontinuation rates and side effects.[23] In response to the public health crisis of opioid misuse and opioid use disorder (OUD), the FDA is encouraging the development of abuse-deterrent formulations (ADFs) of opioid analgesics[24] and alternative modalities for pain management.[1] Additionally, access to medications that treat opioid dependence, such as buprenorphine and naltrexone, has been facilitated and expanded for increasing numbers of patients by programs such as the Drug Addiction Treatment Act of 2000 (DATA 2000).[1]

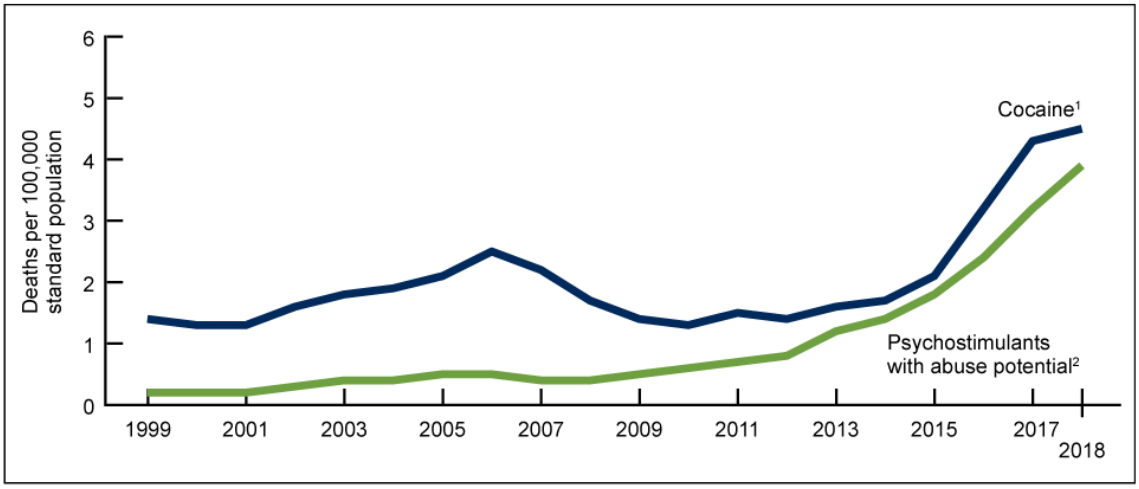
1.2 Cocaine and Methamphetamine

The drug category of psychostimulants includes prescription psychostimulants and illegal drugs like methamphetamine and cocaine. All of these drugs hold the potential

for abuse and addiction. Psychostimulants increase monoamines in the brain. Specifically, methamphetamines and cocaine induce the rapid release of the neurotransmitter dopamine in areas of the brain related to reward pathways, creating a euphoric sensation and reinforcing drug-taking behavior patterns.[25][26] The effects of methamphetamines are felt by the user very quickly after taking the drug, but they wear off quickly as well. These effects can often lead to a cycle of taking repeated doses.[25] Cocaine produces a dopamine flood, which lingers in the neuronal axes. These cells adapt to the amount of dopamine present, decreasing their sensitivity to it. This creates a blockage in cell communication, a state in which cocaine must be taken more frequently or in higher doses to obtain the same high.[26]

Psychostimulants are a rapidly growing concern for public health officials. Rates for cocaine overdose deaths have been climbing since 2012, with the most significant increase between 2016 and 2017 claiming 14,000 lives, a 34% rise.[27] The rates of overdose deaths due to psychostimulant drugs, not including cocaine, have been rising since 2010. The largest increase in this category was again from 2016 to 2017, with a 37% increase, equating to 10,000 deaths.[27] Although opioids have been and will continue to be the focus of the drug crisis in the U.S., there is mounting evidence of a psychostimulant crisis as well. Currently, there are no FDA-approved methods for treating psychostimulant addiction, and behavioral therapy is the only approach used.

Figure 4. Age-adjusted drug overdose death rates involving stimulants, by type of stimulant: United States, 1999–2018



[1]Significant increasing trend from 1999 through 2006, decreasing trend from 2006 through 2012, and increasing trend from 2012 through 2018 with different rates of change over time, $p < 0.05$.
[2]Significant increasing trend from 1999 through 2005, 2008 through 2012, and 2012 through 2018 with different rates of change over time, $p < 0.05$.
NOTES: Deaths are classified using the *International Classification of Diseases, 10th Revision*. Drug-poisoning (overdose) deaths are identified using underlying cause-of-death codes X40–X44, X60–X64, X85, and Y10–Y14. Drug overdose deaths involving selected drug categories are identified by specific multiple-cause-of-death codes: cocaine, T40.5; and psychostimulants, T43.6. Deaths may involve multiple drugs. The percentage of drug overdose deaths that identified the specific drugs involved varied by year, with ranges of 75%–79% from 1999 through 2013 and 81%–92% from 2014 through 2018. Access data table for Figure 4 at: https://www.cdc.gov/nchs/data/databriefs/db356_tables-508.pdf#4.
SOURCE: NCHS, National Vital Statistics System, Mortality.

Figure 1.3: Psychostimulant overdoses in the U.S., 1999 - 2018.

Adapted from the Center for Disease Control and Prevention.[28]

1.3 Opioid Addiction

1.3.1 Behavioral Mechanisms of Addiction

Drug addiction is defined as an uncontrolled craving for a substance and is manifested in drug-seeking behaviors.[29] In the absence of pain, taking opioid drugs causes feelings of pleasure, triggered by the same pathways that promote essential life functions such as eating. This pathway is the mesolimbic reward system.[30] Signals in the ventral tegmental area cause the release of dopamine in the nucleus accumbens.[31] Other areas of the brain contribute to the creation of conditioned associations. These are memories that associate feelings of pleasure with the environment and conditions in which the feeling occurred. This feeling and the memory associated with it can entice individuals to keep using the drug, especially when reexposed to the environment in which the conditioned association was created. Memories and conditioned associations may even lead to more extreme drug-seeking behaviors and, eventually, addiction due to cravings. The feeling of pleasure is one of the initial factors which lead to the progression of drug abuse and addiction, but

soon tolerance and dependence become the stronger motivational forces.[30][32]

Opioid dependence occurs when the brain no longer functions normally without the presence of exogenous opioid. The long-term usage of opioids leads to dependence, which is achieved once a person experiences symptoms of withdrawal when there is no exogenous opioid in their system.[33] Opioid dependence and withdrawal occur due to changes in the locus ceruleus, which is at the base of the brain and produces noradrenaline.[29] This neurotransmitter stimulates wakefulness, breathing, and blood pressure. Upon opioid binding to MORs in the locus ceruleus, noradrenaline release is suppressed. Diminished levels of noradrenaline induce a state of drowsiness, slowed breathing, and lowered blood pressure, typically symptoms desired by individuals with OUD.[29]

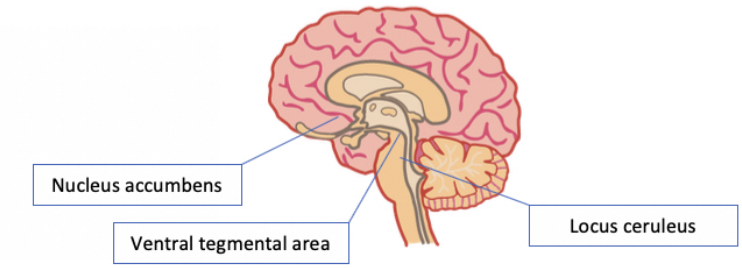


Figure 1.4: Brain areas associated with opioid addiction.

Brain illustration adapted from Project Neuron.[34]

Opioid tolerance occurs when increasing doses of the opioid are required to achieve the same cellular response and experience of pleasure. This effect occurs by the desensitization of neurons and has been linked to the internalization of opioid receptors.[7] Opioid tolerance can be characterized by a rightward shift in the dose-response curve of opioids in tolerant subjects due to pharmacokinetic, pharmacodynamic, or conditioning mechanisms (Figure 1.5).[29] Opioid tolerance occurs because opioid-dependent neurons have increased activity levels, thus increasing the amount of noradrenaline released. Without opioids present, the high activity of these neurons produces the jittery, anxious symptoms often associated with withdrawal.[30] Upon exposure to opioids, the depressive symptoms are lessened, leading to a normal feeling. Withdrawal symptoms can only occur once a patient has developed opioid dependence.[30]

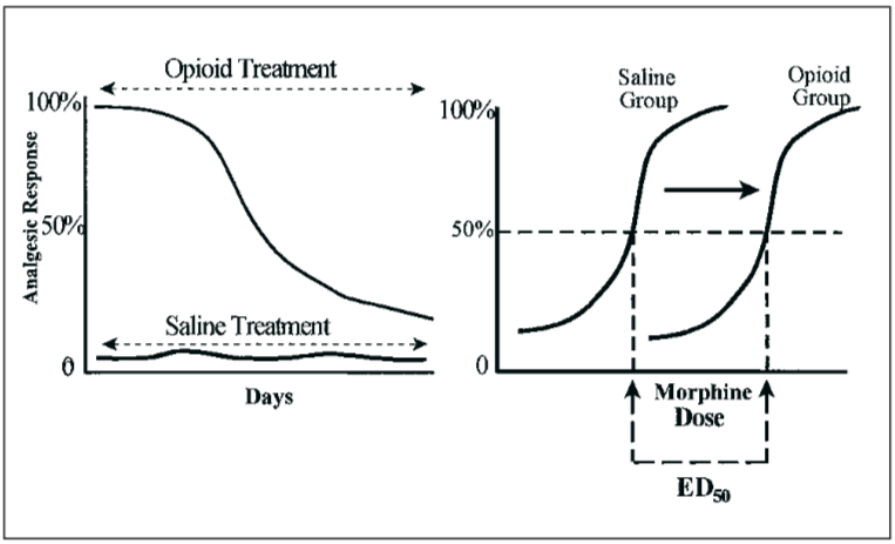


Figure 1.5: Effects of opioid tolerance on dose-response curve.

The dose-response curve for the opioid action obtained following analgesic opioid treatment with morphine shows a right-ward shift, and there is an increase in the medium effective dose (ED_{5_0}) value. Adapted from Pain Research and Management.[35]

Once an individual is dependent upon opioids, a defining factor that keeps them chronically using the drug is withdrawal, symptoms of which begin within 24 hours of the cessation of use in most cases.[29] Withdrawal symptoms last for up to 10 days, decreasing in severity over time. Symptoms include cramps, diarrhea, rhinorrhea, sweating, elevated heart rate, and increased blood pressure, irritability, dysphoria, hyperalgesia, and insomnia.[29] The avoidance of this withdrawal syndrome perpetuates the addiction cycle, increasing the degrees of tolerance and dependence.

1.3.2 Molecular Mechanisms of Addiction

When a ligand binds to an opioid receptor, a conformational change in the transmembrane protein allows for G proteins to couple to the receptor intracellularly. These G proteins are heterotrimeric, with G_α and $G_\beta\gamma$ subunits. Upon coupling to the C-terminus of the opioid receptor, the $G_\beta\gamma$ subunit dissociates from the G_α subunit as GDP is phosphorylated to GTP at the G_α subunit.[36] The G_α subunit inhibits adenylyl cyclases and cAMP production, while $G_\beta\gamma$ subunits interact with transmembrane ion channels.[36] Opioid receptors attenuate the excitability of neurons by modifying pre- and postsynaptic

calcium ion channels and adjusting the inflow of Ca^{2+}.[37][38] All three classes of opioid receptors may also reduce the release of pronociceptive neuropeptides, thus reducing the sensation of pain.[39] The activation of opioid receptors also causes G protein-coupled potassium ion channels to open, preventing the excitation of neurons and halting action potentials.[36] The phospholipase C (PLC)/phosphokinase C (PKC) pathways can also be activated, affecting Ca^{2+} channel activities. All together, the multitude of effects of the activation of opioid receptors lead to decreased transportation of nociceptive signals and thus a significant reduction in the sensation of pain.[36] The activation pathway of opioid receptors is shown below in Figure 1.6.

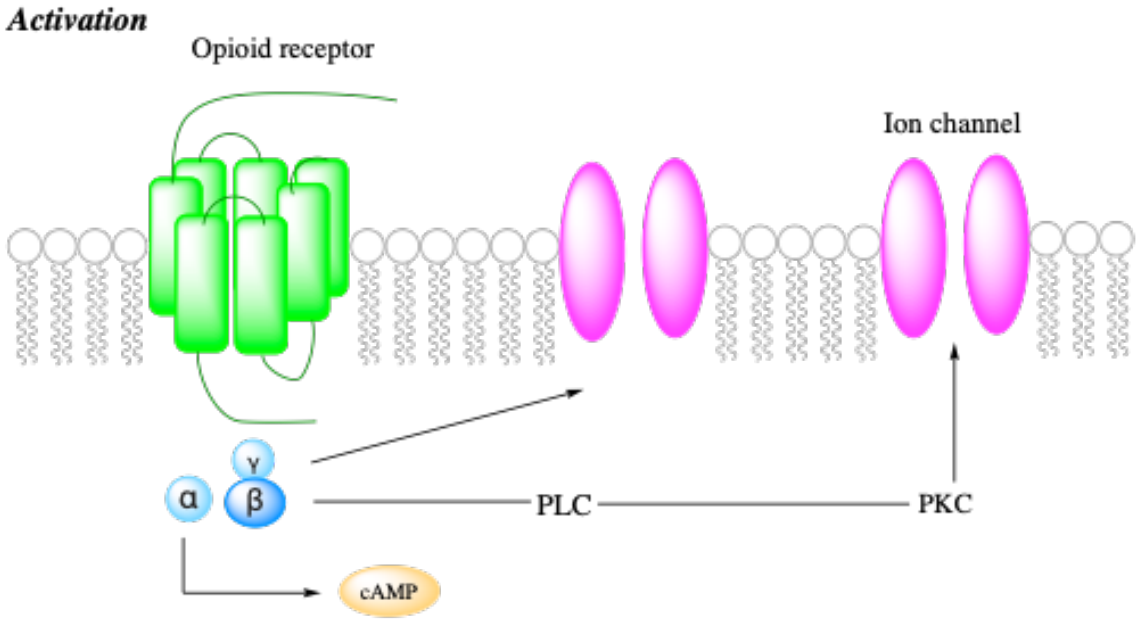


Figure 1.6: Mechanism of opioid receptor activation.

Adapted from *Opioid Treatment of Chronic Nonmalignant Pain*[7]

Kinases phosphorylate intracellular regions of the opioid receptors, and specifically GPCR kinases encourage the binding of arrestin molecules. These arrestin complexes lead to opioid receptor desensitization by the prevention of G protein coupling at the cell membrane. Additionally, internalization of arrestin-bound opioid receptors is promoted as another means of desensitizing the cell to opioid receptor ligands. Dephosphorylated opioid receptors are either reprocessed back into the cell membrane, which reestablishes normal signal transduction, or targeted by lysosomes for degradation.[7] This opioid receptor desensitization pathway is shown below in Figure 1.7.

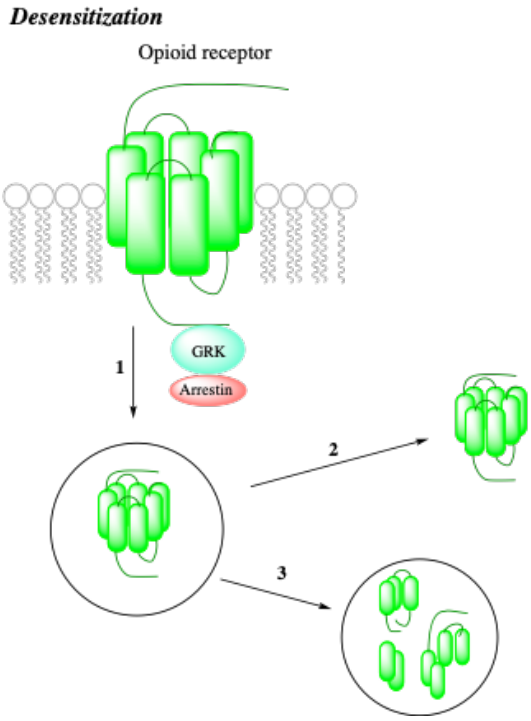


Figure 1.7: Mechanism of opioid receptor desensitization.

1. Arrestin-bound receptors are internalized via a clathrin-dependent pathway and are either **2.** recycled to the cell surface or **3.** degraded by lysosomes.[7]

1.4 Opioid Receptors

It is crucial to discuss and understand the function of opioid receptors (ORs) and the pathways which lead to their activation. The structures of OR ligands, the agonistic/antagonistic effects of these ligands, and their mechanisms for either mitigating or enhancing addiction is crucial in finding viable treatment options for opioid and psychostimulant addiction and withdrawal symptoms. It is the purpose of this study to identify and explain structure-function models of interaction between the kappa opioid receptor and antagonists.

1.4.1 Types of Opioid Receptors

There are three classical opioid receptors: mu (MOR), delta (DOR), and kappa (KOR). These opioid receptors are primarily expressed by central and peripheral neurons, but immune, neuroendocrine, and ectodermal cells have also been shown to express these

receptors.[36] Opioid receptors are transmembrane G protein-coupled receptors (GPCRs), belonging to the subgroup of class A gamma GPCRs.[36]

Drugs which affect opioid receptors are broken up into two categories: agonists, which activate the receptor, and antagonists, which block receptor activity. There are known agonists and antagonists for each classical opioid receptor type. Often, agonists of the three opioid receptors have analgesic properties, but with that often comes addictive properties. An example of this is morphine, an analgesic and agonist of the MOR which is known to be addictive. Fentanyl, another MOR agonist, is a synthetically more potent analog of morphine and is extremely addictive. Antagonists of the ORs are commonly studied as therapies to mitigate addiction and relieve withdrawal symptoms.

This study focuses on the effects of kappa opioid receptor antagonists. Not only is the KOR involved in the sensation of pain and antinociception, but it is also involved in behavioral processes such as depression, anxiety, and addiction.[40] This is possible due to the KOR's ability to relieve the physiological effects of stress. High levels of stress and the use of drugs that act on opioid receptors, which often go hand in hand, increase the production and release of a KOR agonist ligand, Dynorphin.[41] This increased activation of the KOR is known to be related to depressive and addictive states.[31] It has been shown through multiple studies that antagonism of the KOR leads to reduced drug reinstatement due to stress states.[31],[42] Additionally, it has been found to decrease the negative symptoms of withdrawal such as cramping, diarrhea, and anxiety.[33] This antagonistic effect on depressive and addictive states has also been confirmed in cases of cocaine use.[31],[43] Due to the similarities in mechanisms of cocaine and methamphetamines, we hypothesize that KOR antagonism will have potential for treatment of psychostimulants.

1.5 Current Kappa Opioid Receptor Antagonists

Presently, KOR antagonists that are relatively potent and selective have been identified and entered into clinical trials. Due to issues such as long half-lives and adverse side effects, these agents are unlikely to be developed for clinical applications. Two of these compounds, UPHIT and DIPPA, bind irreversibly to the KOR and thus can block the

action of KOR agonists for months.[44] Interestingly, studies involving DIPPA have shown anxiolytic results in rats, highlighting the potential of KOR antagonism for psychological disorders like anxiety and depression.[45]

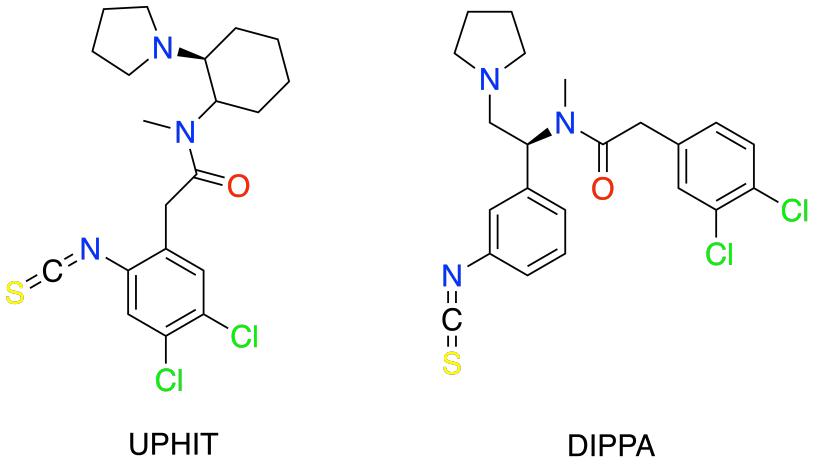


Figure 1.8: Structures of UPHIT and DIPPA.

Other known KOR antagonists like JDTic, norBNI, and GNTI have relatively high potencies and selectivities.[44] However, they have clinically severe drawbacks, including a long delay before exerting their effects, a period of effect of multiple weeks at even the lowest doses, limited brain penetration, and adverse side effects.[46] JDTic especially has been efficacious in preclinical therapeutic models for studying opioid withdrawal, stress-induced cocaine relapse, nicotine withdrawal, depression, and anxiety.[46],[47],[48],[49],[50] Clinical trials were performed evaluating JDTic for the treatment of cocaine abuse, but testing was discontinued after adverse events, including ventricular tachycardia.[46] NorBNI and GNTI have been found to have antidepressant-like effects in the FST (Forced Swimming Test) which modulates stress.[47] GNTI has poor bioavailability, limiting its utility further.[51]

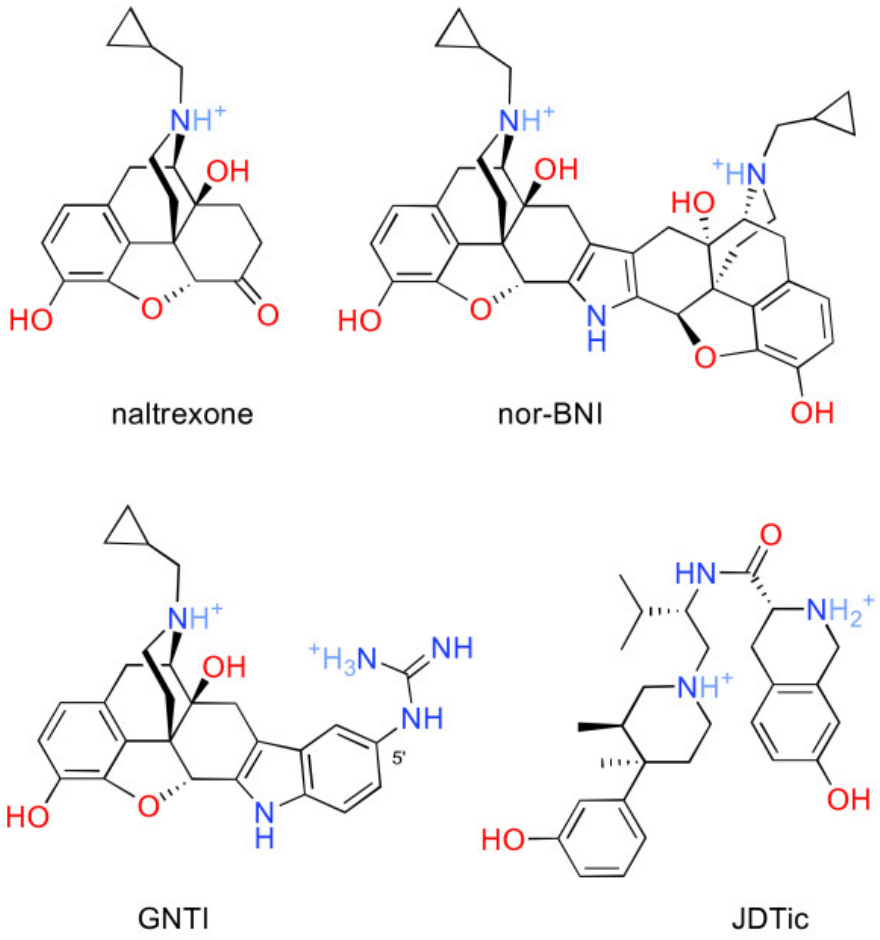


Figure 1.9: Structures of naltrexone, norBNI, GNTI, and JDTic.

Adapted from BMC Pharmacology and Toxicology.[52]

Shorter-acting KOR antagonists with more rapid absorption show more clinical promise. Currently, the shortest-acting agent is CERC-501, with a half-life of 38.5 hours.[44] This compound has been shown to reverse the analgesic effects of KOR agonists.[46] CERC-501 shows therapeutic effects in preclinical trials of alcoholism and also shows antidepressant-like effects in the FST, like norBNI and GNTI.[46],[53],[54] Another agent, PF-4455242, has exhibited similar properties and effects with promise for the treatment of OUD and related disorders. However, there has been evidence of toxicity in animal studies when taken for over 90 days.[46],[55] The final compound in this class is AZ-MTAB, which blocks agonistic effects on the KOR in animal models.[44],[56]

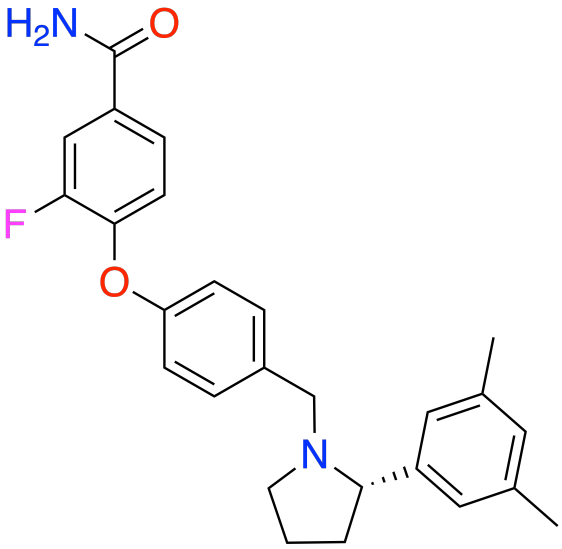


Figure 1.10: Structure of CERC-501.

Buprenorphine is a drug currently used to treat opioid dependence as a weak partial MOR agonist, KOR and DOR antagonist, and weak partial agonist for nociceptin receptors.[57] Because it is not a selective KOR antagonist, its therapeutic effects cannot be linked conclusively to KOR antagonism. The combination of buprenorphine with naltrexone, a nonselective OR antagonist, has allowed for the creation of a relatively selective KOR antagonist.[58] This combination proves to be a potential method for the treatment of cocaine abuse and the prevention of relapse in both cocaine and opioid-dependent individuals.[59][60] Clinical trials on this subject begin to link the therapeutic activity of buprenorphine with KOR antagonism.[61],[42]

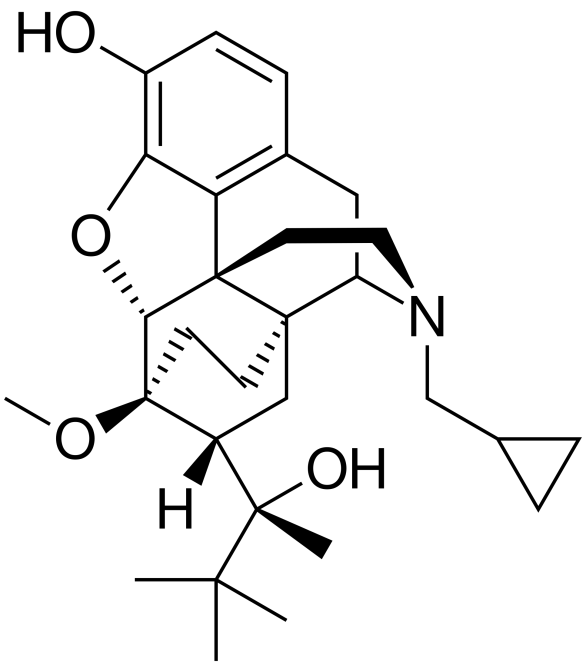


Figure 1.11: Structure of buprenorphine.

Thus, selective, short-acting KOR antagonists are needed to determine if long and persistent action is required to yield desired therapeutic effects and to increase the viability of KOR antagonists for preclinical studies. The agents discussed above all have persistent effects complicating drug development due to unfavorable pharmacodynamic effects.

1.6 Salvinorin A

Salvinorin A is a naturally occurring opioid receptor agonist and is the main active ingredient in *Salvia divinorum*, a hallucinogenic plant native to Mexico.[62] It is highly potent, shows high selectivity and affinity for the KOR, and does not show affinity for other receptors in the body,[63] but has never advanced to clinical trials due to its strong hallucinating effects and short half-life of 16 minutes.[64] This short half-life is partially due to the hydrolysis of the C2 ester by esterases.[65] Salvinorin A has a unique structure (Figure 1.12). Most naturally occurring hallucinogens target the serotonin 5-HT_2a receptor, whereas salvinorin A is highly selective for the KOR.[63] It is different from other KOR agonists because it lacks a basic nitrogen, so it is not ionized at cellular pH. This basic nitrogen was thought to be necessary for binding to an opioid receptor.[66] Qualities of

salvinorin A, such as its hallucinogenic effects and short half-life, contribute to salvinorin A's perception as a poor drug for treating pain. However, its high selectivity for the KOR has made it attractive as a lead compound for drug development.

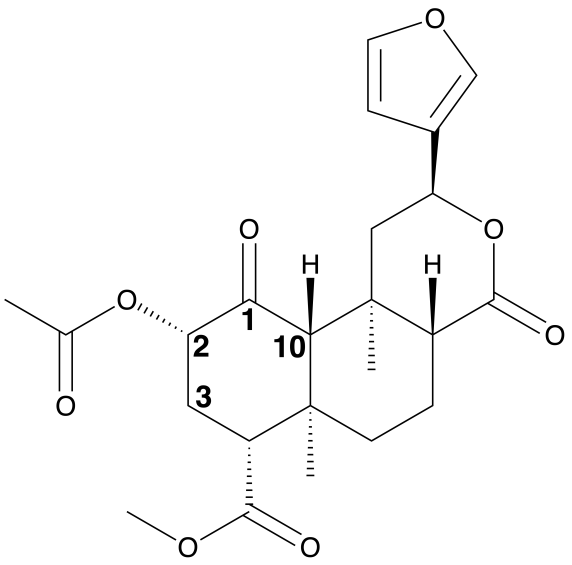


Figure 1.12: Salvinorin A Structure.

1.6.1 Salvinorin-Based Compounds

In literature, there are only six salvinorin-based compounds have shown antagonism against any opioid receptor (**1-6**, Figure 1.13). All display antagonism toward the MOR, DOR, and KOR except **5**, which is an antagonist for the MOR and DOR but has partial agonist activity on the KOR.[67] Each shows varied selectivity to the KOR. All other salvinorin-based compounds thus far have only shown OR agonism. Each of these antagonists show a modification at C1, replacing the ketone functional group with an alcohol, alkene, or methylene. Though these modifications appear small, they result in differential binding to the various ORs. It is expected that the ring undergoes a change in topology when the ketone functional group is lost. Using crystal structures of the active and inactive states of the KOR, interactions with salvinorin-based compounds can be explored using these known antagonists. This study hypothesizes that selective salvinorin-based KOR antagonists can be developed using the active and inactive KOR. These antagonists would have short half-lives, leading to a unique opportunity to study drug abuse, withdrawal, and short-acting KOR antagonists. Coupling structure-based drug design

(SBDD) and computational modeling, more selective salvinorin-based KOR antagonists can be developed while elucidating pertinent interactions in antagonistic functionality at the KOR.

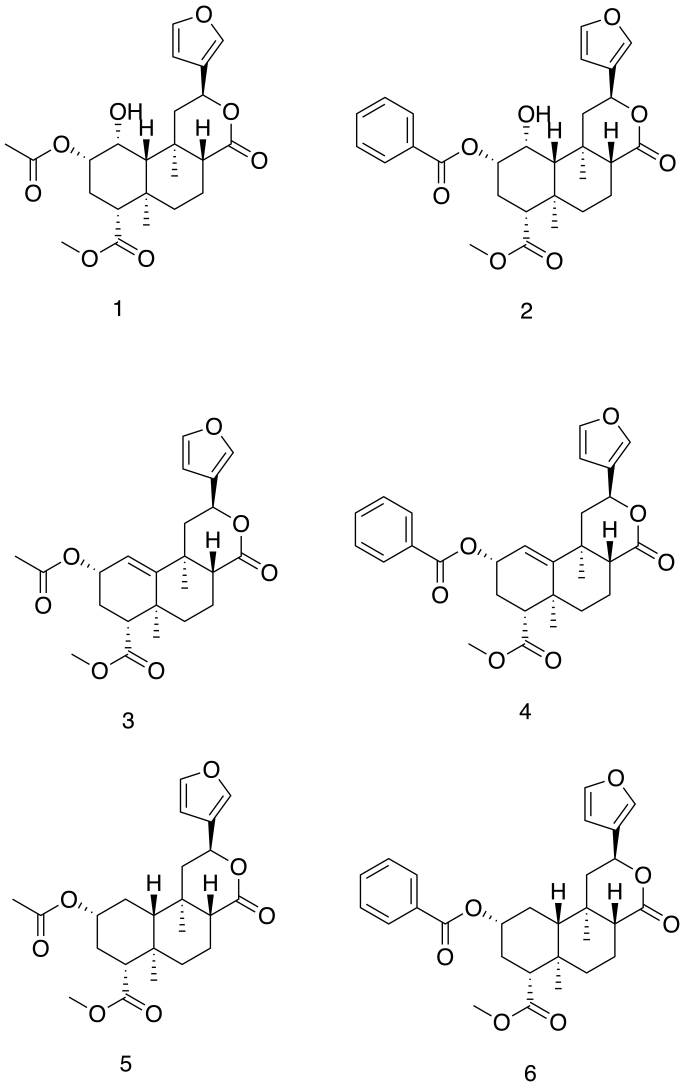


Figure 1.13: Salvinorin-based compounds **1-6**.

In order to test our hypothesis that the ring topology of the salvinorin scaffold is directly related to its agonistic or antagonistic effects on opioid receptors, we proposed and studied three scaffolds (**A-C**, Figure 1.14). The use of these scaffolds in docking procedures will aid in formulating a complete understanding of the structure-activity relationship between salvinorin-based compounds and opioid receptors.

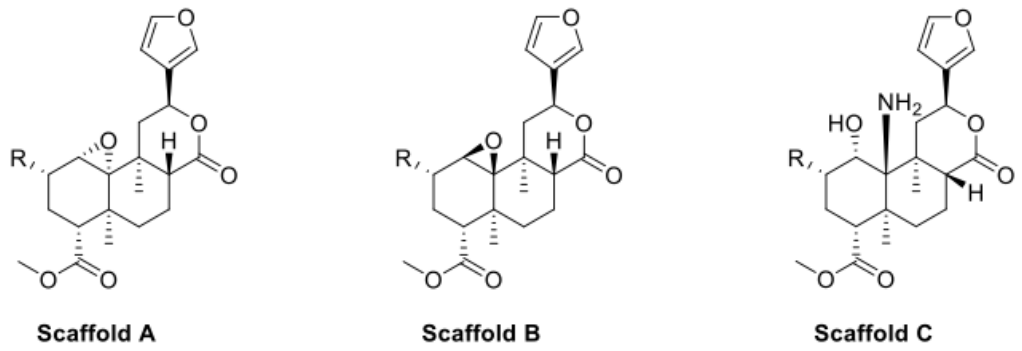


Figure 1.14: Salvinorin-based scaffolds **A, B, and C**.

Currently, no drugs have been approved to target the KOR. This is principally due to limited knowledge of the molecular mechanism of KOR antagonism. The goal of this study is to define necessary interactions for KOR antagonism and novel salvinorin-based antagonists. The elucidation of vital interactions within the KOR will help fulfill unmet needs in understanding the mechanisms of KOR antagonism. This subject is of particular interest because the National Institute on Drug Abuse (NIDA) listed KOR antagonists as one of their "top 10 most-wanted in fighting the opioid crisis".[68] The synthesis of these salvinorin-based compounds was carried out simultaneously in the hopes of future *in vitro* and *in vivo* studies. Thus, this study provides a multifaceted approach involving structure-based drug design (SBDD) and computational methods. The computational methods utilized include docking and molecular dynamics protocols within the active and inactive KOR receptor X-ray crystal structures. This work will help to extend knowledge of the structure-activity relationship of salvinorin-based compounds and elucidate crucial interactions for desired affinity and antagonism. This knowledge can help to mold future salvinorin-based compounds for opioid receptors and help in the creation of new KOR ligands.

Chapter 2

Experimental

2.1 Computational Procedures

All docking protocols were performed using Maestro 19.1, a program which focuses on the conformation energy or Glide scores associated with ligand binding. Glide scores are related to how entropically favored an interaction is. Compounds **1-6** (Figure 1.13) were docked with X-ray crystal structures for the active (PDB:6B73) and inactive (PDB:4DJH) states of the KOR, obtained from the Protein Database (PDB). The proteins were then prepared using the Protein Prep module in Maestro. Preprocessing included adding missing side chains and removing chains B and C as well as any ligands within the receptor. Additionally, the pH of the system was adjusted to 7.4 to mimic the physiological environment. The water molecules within the binding sites were then reviewed, and any waters not involved in binding were removed. In the case of the active KOR, there were no waters within its binding site. In the inactive state, waters were present, so two versions of this protein were carried forward: one with water and one without water. These three versions of the KOR were then optimized.

In order to dock compounds to the optimized KORs, receptor grids were generated. Receptor grids define the binding site within the receptor, focusing the computer on this specific area of the protein. Two methods were used in generating these grids. In cases where an exogenous ligand was co-crystallized, an atom within that ligand was selected. A 20 Å box was defined around this ligand. This was the preferred method. Alternatively,

x,y,z coordinates were provided to create the 20 Å receptor grid. Rotatable groups within 3 Å were defined and the grid was generated. The ligands, compounds **1-6** (Figure 1.13) and scaffolds **A**, **B**, and **C** (Figure 1.14), were drawn, prepared, and 3-D minimized using the LigPrep function in the Schrodinger Suite 2019. Docking was performed using the prepared active state KOR, inactive state KOR with waters, and the inactive state KOR without waters on the standard precision and extra precision protocol settings. The free energies associated with the binding of the docking-preferred complex of the salvinorin-based antagonists and scaffolds were recorded using the Prime-MMGBSA.

2.2 Experimental Procedures

In order to fulfill the SBDD and synthetic portion of this study, we plan to synthesize compounds **1-6** in order to further demonstrate the importance of ring topology of the salvinorin scaffold in KOR selectivity and antagonism. Much needed *in-vitro* testing will be performed upon the successful synthesis of these potential KOR antagonists. At this time, synthesis of these compounds is ongoing. Procedures in the literature for the following synthetic schemes did not provide the same degrees of purity and had lower yields than reported[67], so methods had to be adjusted. We additionally implemented minor changes in the established synthetic route[67] to make the process more efficient.

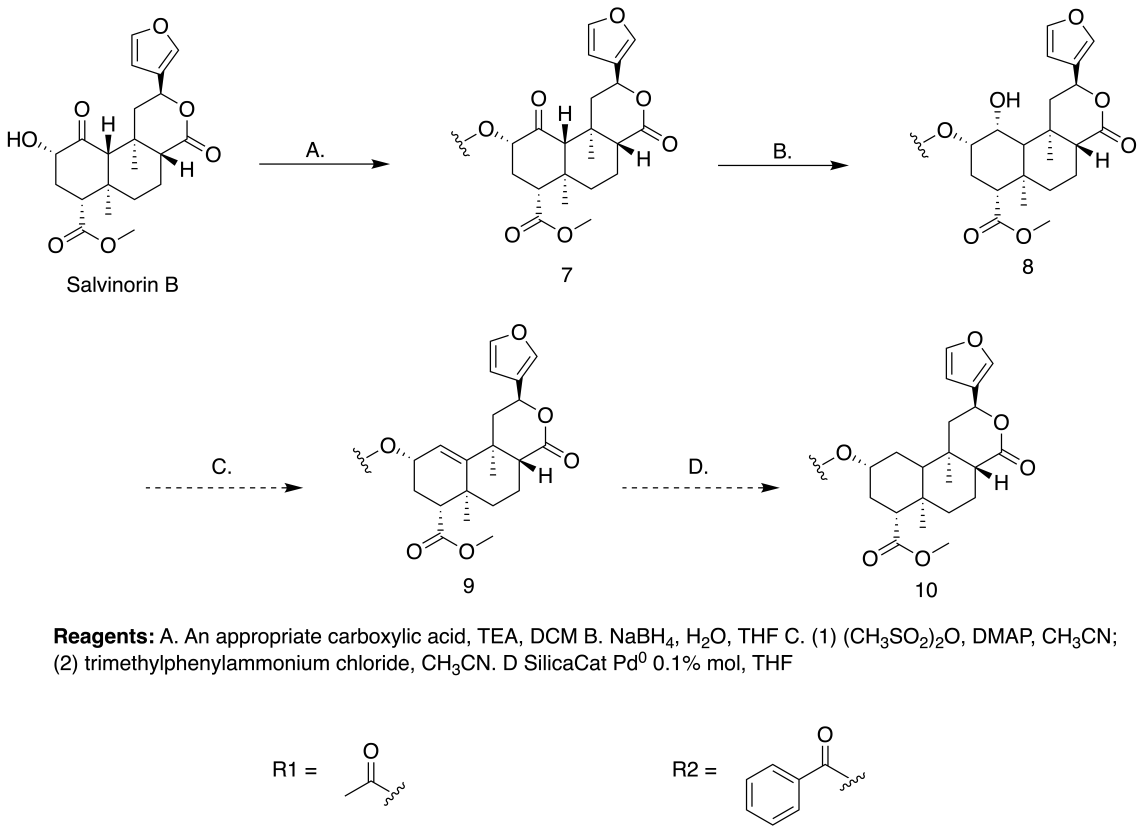


Figure 2.1: Synthetic scheme of salvinorin-based compounds.

2.2.1 Synthetic Step A: Esterification of Salvinorin B

In order to begin the synthesis of salvinorin-based compounds (SBCs) **1,3, and 5**, acetyl chloride was esterified to salvinorin B (Figure 2.2) to afford salvinorin A, whereas to make compounds **2, 4, and 6**, the appropriate acyl chloride, benzoyl chloride, was used to afford **SBC 2a**. These starting materials were prepared using the following procedure: To a solution of salvinorin B (100 mg, 2.56 x 10^{-4} mmol, 1 eq) and TEA (53 μL, 5.12 x 10^{-4} mmol) in 20 mL DCM at 0°C was added acid chloride (5.12 x 10^{-4} mmol, 2 eq) and stirred 2 hours. The DCM was evaporated to afford the final product, which was immediately carried on to the next reaction without further purification.

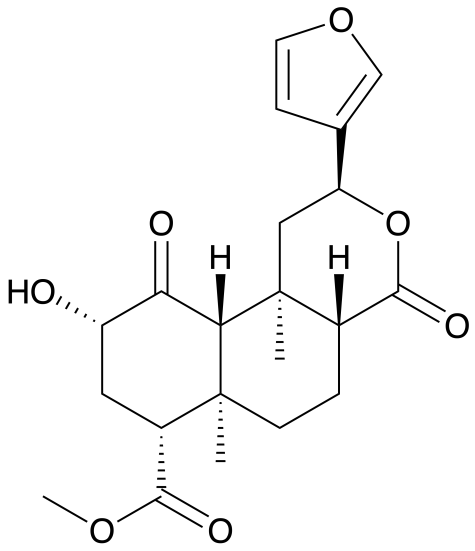


Figure 2.2: Structure of salvinorin B.

2.2.2 Synthetic Step B: Reduction of the C1 Ketone

SBC 1: To a round-bottom flask, THF (2 mL) was added to salvinorin A, (89 mg, 0.205 mmol) and the mixture was magnetically stirred at gentle reflux for 10 min. To this was added an aqueous solution of $NaBH_4$ (38 mg, 1 mmol in 0.3 mL) slowly. After stirring at reflux for 10 min, a second addition of $NaBH_4$ powder was made (1 eq, 7.6 ng, 0.205 mmol) and reflux was continued for 5 min. The reaction mixture was then placed in an ice bath and its progress was checked by TLC. The reaction mixture was diluted with ethyl acetate (EtOAc) (50 mL) and extracted with saturated sodium chloride (2 X 30 mL). The aqueous phases were extracted with EtOAc (2 X 15 mL) and the combined organic phases were dried (Na_2SO_4) and evaporated. Purification of the crude product was done by column chromatography (CH_2Cl_2 with increasing amounts of EtOAc).

SBC 2: A mixture of **SBC 2a** (100 mg, 0.20 mmol) and THF (8 mL) was stirred at reflux for 5 min. An aqueous solution of $NaBH_4$ (38 mg, 1.00 mmol in 4 mL) was added slowly. After stirring at reflux for 10 min, a second addition of aqueous $NaBH_4$ was made (8 mg, 0.21 mmol in 1 mL) and continued to reflux for 5 min. The reaction mixture was then placed in an ice bath and its progress was checked by TLC. The reaction mixture was diluted with ethyl acetate (EtOAc) (30 mL) and extracted with saturated sodium chloride (2 X 30 mL). The aqueous phases were extracted with EtOAc (2 X 15 mL) and

the combined organic phases were dried (Na_2SO_4) and evaporated. Purification of the crude product was done by column chromatography (Hexanes/EtOAc, 6:4).

Note: No results were yielded from the performance of these reactions. The following table summarizes adjustments to the reaction conditions made in order to optimize the reduction of the C1 ketone.

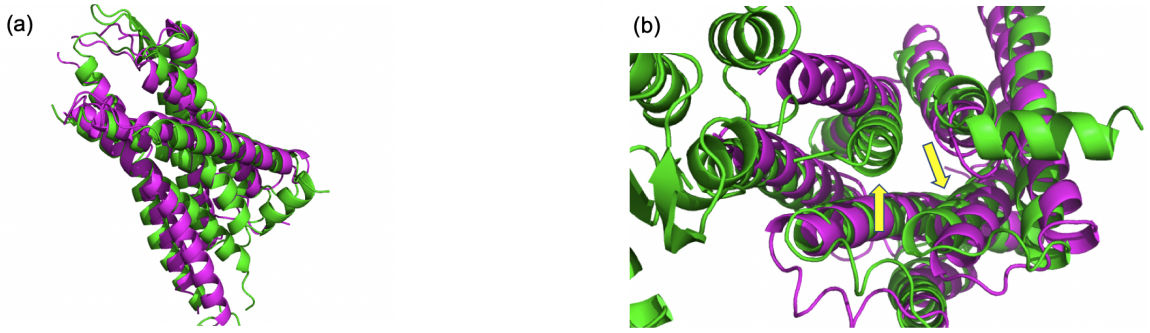
Solvent	Temperature	Reaction Time	Product Yield
EtOH	80 °C	15 minutes	57%
ACN	RT	No reaction occurred up to 4 hours, then o/n.	Yielded a complex mixture. Trace amounts.
THF/EtOH(85/15)	80 °C	30 minutes	10%
DCM/THF(50/50)	-78 °C to RT over 3h.	3 hours	Yielded a complex mixture. Trace amounts.

Figure 2.3: Summary of reaction conditions used in reducing the C1 ketone.

Chapter 3

Results and Discussion

In order to elucidate the interactions of compounds **1-6** and salvinorin scaffolds **A-C** that confer antagonistic effects on the KOR, the docking position and interactions of salvinorin A, an agonist, were studied for means of comparison. Another study determined that Ile316 and Gln115 are important in the binding of salvinorin A to the KOR as an agonist.[69] Asp138 is conserved as a -NH donor and Tyr320 is highly conserved among GCPRs. The aromatic ring and the hydroxyl group of Tyr320 are thought to have a crucial interaction with the methyl ester group at C2 of salvinorin A, in which salvinorin A hydrogen bonds with its hydroxyl group.[69] Tyr313 and Tyr119 play a stabilizing role in the agonist binding conformation, although they are not directly accessible to the salvinorin A ligand. This stabilizing effect may be due to the lack of a strong ionic receptor-ligand interaction between the KOR and salvinorin A.

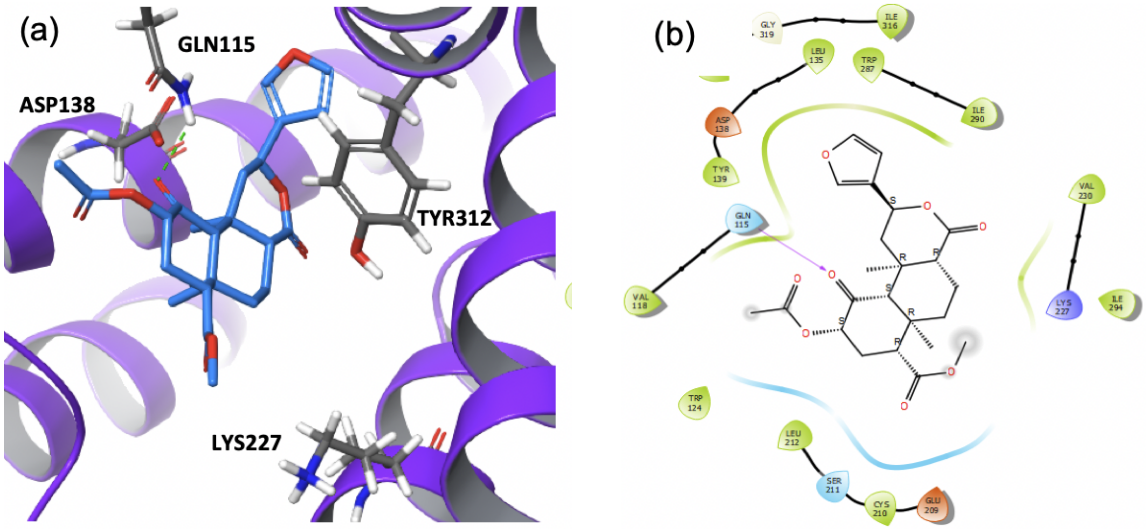


(a) Active Purple) and inactive (Green) states overlay.

(b) Arrows highlight the marked difference of the inactive/active state of the KOR.

Figure 3.1: Active and inactive crystal structures of the KOR

The results of the docking study suggest that the furan ring of the core salvinorin A structure has a different orientation within the binding pocket depending upon whether the KOR was in its active or inactive state (Figure 3.1). The binding of the agonist salvinorin A demonstrates orientation of the furan ring deeper into the KOR to Tyr320 in the KOR active state. The C1 carbonyl exhibits a hydrogen bonding interaction with Gln115 (Figure 3.2). These results confirm the expected docking interactions of salvinorin A in the KOR.[69]

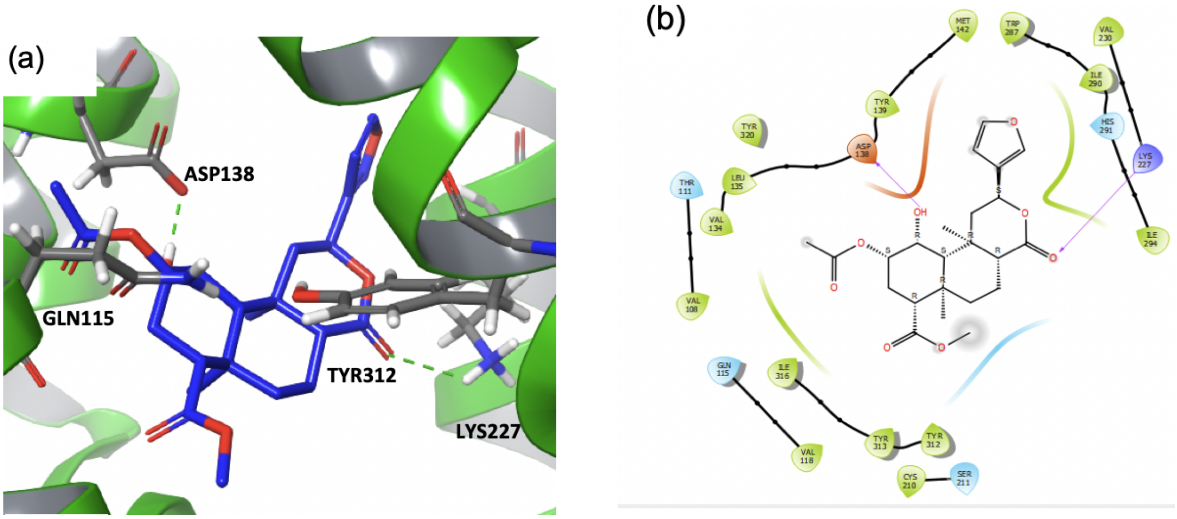


(a) Orientation of salvinorin A (Light Blue) in the active state of the KOR (purple).

(b) 2D orientation of salvinorin A within the active state of the KOR.

Figure 3.2: salvinorin A bound to active KOR.

Next, the known KOR antagonists **1-6** were studied using the same computational procedure. Another study showed that the removal of the C1 ketone causes a 6-fold decrease in agonist activity.[70] In the antagonist bound state, Tyr320 and Lys227 contribute to intrahelical interactions.[69] Our computational study showed that the salvinorin-based antagonists **1-5** protrude less deeply into the binding pocket with their furan rings compared to salvinorin A, leading to decreased interaction with Tyr320 (Figure 3.3). This is due to the closed-state of the inactive binding site compared to that of the active state. The C1 α-hydroxyl hydrogen bonds with Asp138. These interactions are a result of the change in ring topology that occur upon substitution of the C1.



(a) Upward orientation of **SBC 3** (Dark Blue) within the inactive state (Green).

(b) 2D interaction diagram showing key residues that interact with **SBC 3** within the inactive state of the KOR.

Figure 3.3: **SBC 3** bound to inactive KOR.

The docking poses of the salvinorin-based antagonists differ accordingly with the change from active/inactive state of the KOR, corresponding with increased space in the active state (Figure 3.1). Thus, in the active state, salvinorin A's furan rings extend farther into the receptor than the antagonists (Figure 3.4). This pose puts the ligand in a position oriented toward residues known to be critical in KOR selectivity: Val108, Val118, Ile294, and Tyr 312.[69] In the inactive state, **SBC 3** may have a hydrogen bonding interaction with Lys227, securing the ligand in place. The other salvinorin-based scaffolds exhibited similar binding poses to **SBC 3** (Figure 3.6).

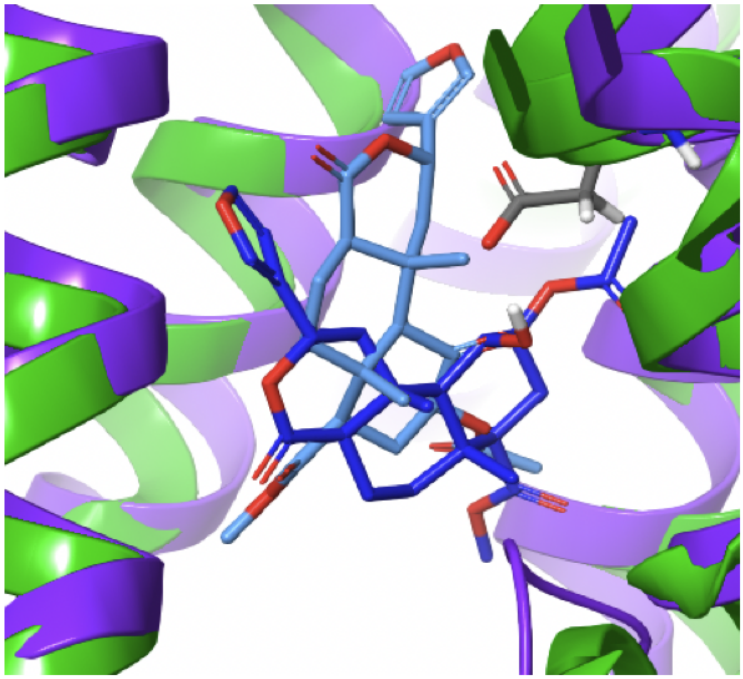


Figure 3.4: Overlay of salvinorin A and **SBC 3**

Overlay of salvinorin A (Light Blue) in the active state (Purple) and **SBC 3** (Dark Blue) in the inactive state (Green).

We hypothesized that modifying the SBCs at the C3 position to optimize the number and strength of the interactions with nearby hydrogen-bond eligible residues would increase selectivity for the KOR over the MOR and DOR. This strategy involves increasing lipophilic interactions with Val 118. Unfortunately, the synthesis of these compounds would be arduous, especially considering stereospecificity. Thus, substitutions at C10 were considered, which could provide additional hydrogen bonding and electrostatic interactions with Asp138 and Tyr312 for a similar effect in increasing selectivity. This hypothesis was confirmed computationally, yielding improved Glide scores (Figure 3.5).

Compounds	Glide Score(kcal/mol)	Glide Emodel (kcal/mol)	ΔG (kcal/mol)
1	-4.676	-53.717	-46.07
2	-4.521	-52.082	-46.97
3	-2.925	-40.682	-32.40
4	-3.760	-50.647	-46.93
5	-4.476	-37.249	-33.47
6	-4.520	-52.798	-44.30
Scaffold A, R-methyl acetate	-4.199	-46.089	-33.94
Scaffold B, R-methyl acetate	-4.672	-49.459	-35.86
Scaffold C, R-methyl acetate	-5.074	-53.313	-39.57

Figure 3.5: Docking results of **SBCs 1-6** and scaffolds **A-C**

Taken together, the results of this study provide insights into the docking poses of KOR antagonists. These antagonists have a different pose compared to that of salvinorin A, which can be exploited by varying the ring topology of salvinorin A with substitutions. Changing the topology of the core structure of salvinorin A thus determines whether the compound will bind to the active or inactive state of the KOR. Upon binding to the active state, it has been shown that the furan ring of agonists reach more deeply into the receptor. The designed scaffolds have similar poses to the known KOR antagonists, which are stable, as suggested by their ΔG scores (Figures 3.5 and 3.6).

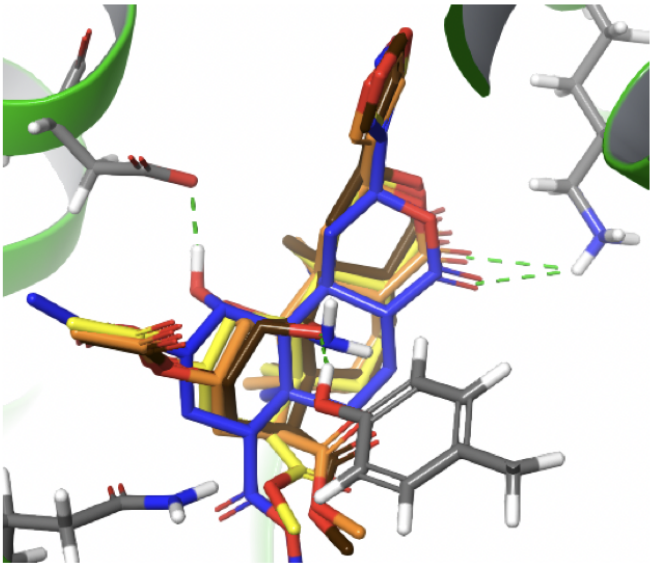


Figure 3.6: Overlay of salvinorin-based scaffolds and **SBC 3**.

Overlay of **SBC 3**, scaffold **A** (Orange), scaffold **B** (Brown), and scaffold **C** (Yellow) in the KOR inactive state.

Chapter 4

Conclusion

Our approach of coupling structure-based drug design with computational docking methods has provided insights into the structure-function relationship between salvinorin-based antagonists and the KOR. The antagonists studied were salvinorin-based compounds **1-6** and scaffolds **A-C**, novel potential KOR antagonists.

According to the docking study, the topology of the tricyclic core of the salvinorin structure plays a role in the activity of KOR antagonists. The tricyclic core undergoes this change in topology due to the loss of the C1 ketone, changing the molecular geometry at C1 from trigonal planar to tetrahedral. The reduction in the bond angle in the hexane ring causes a shift in the topology of the entire core ring structure of salvinorin. This finding has allowed for the design of novel salvinorin-based scaffolds to antagonize the KOR. In order to increase selectivity for the KOR, C10 was chosen as the site of substitution. C10 was the best candidate due to its synthetic viability, and substitutions were proposed to increase favorable interactions with nearby residues. These scaffolds exhibited antagonism on the KOR with increased selectivity according to their glide scores, affirming the hypothesis that ring topology plays a role in the antagonistic effect on the KOR.

Additionally, a critical residue, Lys227, was identified as key for the binding of antagonists within the active state of the KOR. We believe that this hydrogen bonding interaction acts as an anchor holding the ligand in place. Thus, this study successfully identified pertinent interactions for the antagonism of the KOR.

The synthetic portion of this study is still in progress. Upon its successful completion, we plan on obtaining *in vitro*, specifically IC50 analysis, and *in vivo* testing for these compounds to clarify the action and binding of these salvinorin-based agents with opioid receptors.

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